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Osmotically induced cytosolic free Ca^{2+} changes in human neutrophils

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Abstract

Cytosolic free Ca^{2+} concentration in neutrophils was measured by ratiometric fluorometry of intracellular fura2. Increasing the extracellular osmolarity, by either NaCl (300–600 mM) or sucrose (600–1200 mM), caused a rise in cytosolic free Ca^{2+} ($\Delta_{max} \cong 600$ nM). This was not due to cell lysis as the cytosolic free Ca^{2+} concentration was reversed by restoration of isotonicity and a second rise in cytosolic free Ca^{2+} could be provoked by repeating the change in extracellular osmolarity. Furthermore, the rise in cytosolic free Ca^{2+} concentration occurred in the absence of extracellular Ca^{2+}, demonstrating that release of intracellular fura2 into the external medium did not occur. The osmotically-induced rise in cytosolic free Ca^{2+} was not inhibited by either the phospholipase C-inhibitor U73122, or the microfilament inhibitor cytochalasin B, suggesting that neither signalling via inositol tris-phosphate or the cytoskeletal system were involved. However, the rise in cytosolic free Ca^{2+} may have resulted from a reduction in neutrophil water volume in hyperosmotic conditions. As these rises in cytosolic Ca^{2+} ($\Delta_{max} \cong 600$ nM) were large enough to provoke changes in neutrophil activity, we propose that conditions which removes cell water may similarly elevate cytosolic free Ca^{2+} to physiologically important levels.

Keywords: Neutrophil; Cytosolic free Ca^{2+} concentration; Osmolarity; Cystic fibrosis; Signalling; Imaging; Cell shape; (Lung damage); (Inflammation)

1. Introduction

Unlike most cells of the body which are associated with specific locations in the body, neutrophils migrate from the blood stream to various parts of the body, including the kidneys, lung and gut. In so doing, they may be exposed to varying osmotic conditions. For example, in the renal medulla of the kidney neutrophils experience increased osmolarity, up to 1400 mosmol [1,2]. Neutrophils also experience raised osmotic conditions in the interstitial fluid of enclosed inflammatory spaces, where hydrolysis of macromolecules occurs [3,4]. In order to understand the physiological and pathological roles of neutrophils within the body, it is therefore important to establish whether changes in osmolarity influence neutrophil behaviour. Previous studies addressing this point have reported that elevated osmolarity affects exocytosis [5], chemotaxis [6] and oxidase activation [7] by the neutrophils. However, the mechanism by which these effect occurred have not been fully established. In a classic series of papers, Chandler and co-workers demonstrated that hyperosmotic conditions inhibited exocytosis and that the Ca^{2+}

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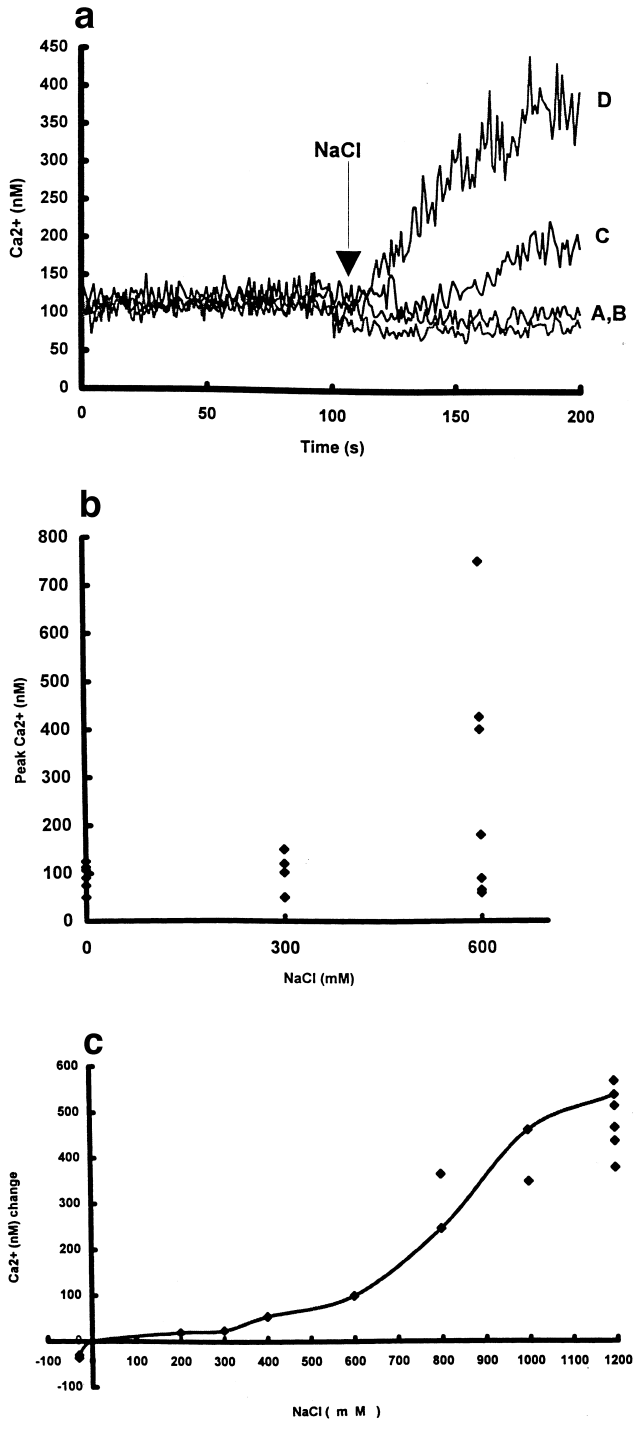
signal induced by f-Met-Leu-Phe was modified under these conditions [5,8]. As it is now known that Ca^{2+} signalling underlies a number of neutrophil responses [9], we have investigated the direct effect of increasing osmolarity on neutrophil cytosolic free Ca^{2+} concentration. We report here that cytosolic free Ca^{2+} is elevated in response to large increases in osmolarity and that these are correlated with the accompanying reduction in the volume of the neutrophil.



2. Materials and methods

2.1. Neutrophil isolation

Neutrophils were isolated from heparinised blood of healthy volunteers as described previously [10]. Following dextran sedimentation, centrifugation through Ficoll-Paque (Pharmacia) and hypotonic lysis of red cells, neutrophils were washed and resuspended in Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM Hepes and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH).

2.2. Measurement and imaging of cytosolic free Ca^{2+} concentration and cell size

Neutrophils were loaded with fura2 and population and imaging measurements performed as previously described [11,12]. Excitation wavelengths were achieved by using a Spex Fluorolog dual wavelength fluorimeter (Glen Spectra, Stanmore, UK) and ratio images acquired by an intensified IC-200 CCD camera (PTI) coupled to Nikon microscope and a rapid wavelength switching monochromator analysis system (Photon Technologies International, Surbiton, UK) [11,12]. The cell size was defined from the 340/380 nm ratio image using a thresholding algorithm so that this image corresponded to the edge

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Fig. 1. Effect of salinity on cytosolic free Ca^{2+} concentration. (a) The change in cytosolic free Ca^{2+} concentration in human neutrophils induced by the addition of NaCl, A = 200 mM, B = 300 mM, C = 400 mM and D = 600 mM (added at the arrow), is shown. (b) The peak change in cytosolic free Ca^{2+} concentration in neutrophils from individual donors for the NaCl concentration shown. (c) The relationship between NaCl concentration and cytosolic free Ca^{2+} concentration is shown. The data are from six individual donors. The neutrophils from each donor were subjected to both 600 mM and 12 000 mM NaCl and at least one other concentration of NaCl. In each case, the changes have been normalised relative to that for 600 mM NaCl.

of the cell as viewed by phase contrast. This enabled cytosolic free Ca^{2+} concentration and cell size to be measured simultaneously and calculated from the same image data set. The cytosolic free Ca^{2+} concentration was calculated as the mean pixel value within an area which excluded the actual cell edge as this was often contaminated with artefactual ratiometric values resulting from the low fura2 intensity in this region [12].

3. Results

3.1. Increased extracellular salinity elevates neutrophil cytosolic Ca^{2+}

In order to establish whether changes in extracellular NaCl concentration had any effect on the cytosolic free Ca^{2+} concentration in neutrophils, fura2-loaded cells were exposed to extracellular media of altered salinity. Reducing NaCl concentration by 25% (to 90 mM NaCl), resulted in a cytosolic free Ca^{2+} decrease of approximately 50 nM. However, increasing the concentration of NaCl (above 300 mM) caused a rapid, long-lived rise in cytosolic free Ca^{2+} concentration (Fig. 1a). Within 10 s of increasing the extracellular salinity, the cytosolic free Ca^{2+} concentration began to rise and continued at a rate of approximately 5–10 nM/s. The extent of the cytosolic free Ca^{2+} rise was dependent on the donor (Fig. 1b) and was related to the change in salinity, reaching a maximum at 1200 mM (Fig. 1c). In NaCl concentrations of 300–600 mM, the cytosolic free Ca^{2+} concentration rose significantly above the resting level, from approximately 100 nM at rest to in excess of 400 nM. This cytosolic free Ca^{2+} concentration would be sufficient to have physiological consequences in the neutrophil, and is above the threshold for triggering both oxidase activation and exocytosis of granular contents [13,14]. It was concluded that increased extracellular salinity triggered physiologically significant changes in cytosolic free Ca^{2+} concentration within human neutrophils.

3.2. The effect of salinity is due to its osmolarity

Neutrophils are known to be freely permeable to Cl^- ions and are not expected to regulate their cytosolic Cl^- concentration [15]. In order to test whether the effect of increasing salinity was due to the increase in intracellular Cl^- ion concentration, the effects of another sodium salt and of changing the osmolarity without changing the Cl^- ion concentration were established. Na acetate and sucrose each elicited concentration-dependent rises in cytosolic free Ca^{2+} concentration (Fig. 2a) of similar magnitude to NaCl when used at the equivalent concentration (Fig. 2b). These data therefore indicate that

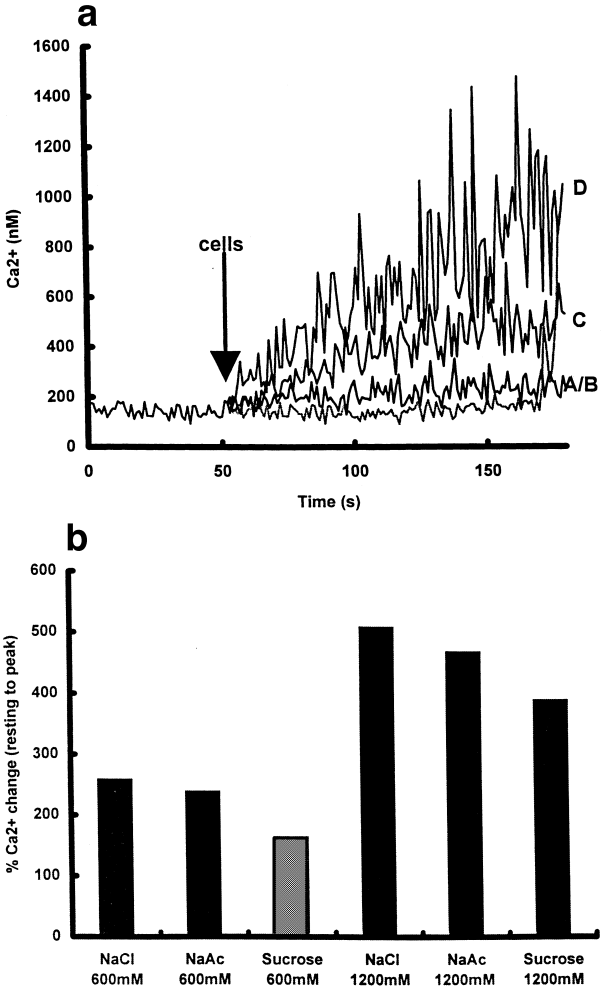

Fig. 2. Effect of osmolarity on cytosolic free Ca^{2+} concentration. (a) The change in cytosolic free Ca^{2+} concentration in human neutrophils induced by the addition of sucrose, A = 600 mM, B = 800 mM, C = 1000 mM and D = 1200 mM (added at the arrow), is shown. (b) The peak change in cytosolic free Ca^{2+} concentration in neutrophils induced by NaCl, Na acetate and sucrose at the concentration shown. The data are representative of at least three experiments on different donors.

Cl^- ions was not required for the effect on cytosolic free Ca^{2+} concentration.

3.3. The rise in cytosolic free Ca^{2+} was not due to irreversible cell damage

It was possible that the increase in extracellular osmolarity caused cell breakage and the release of intracellular fura2 into the extracellular medium. This would have resulted in changes in the saturation of fura2 with Ca^{2+} which would have been indistinguishable from changes in cytosolic free Ca^{2+} concentration. In order to determine whether cell breakage contributed to the measured change in fura2 signal, three characteristics of the signal were assessed: (i) its dependency on extracellular Ca^{2+}, (ii) its reversibility on the restoration of osmolarity, and (iii) its intracellular or extracellular location (by direct visualisation).

3.3.1. Cytosolic free Ca^{2+} rises in the absence of extracellular Ca^{2+}

A crucial test of whether the increase in fura2 signal resulted from release of fura2 into the extracellular medium was to determine whether it occurred in the absence of extracellular Ca^{2+}. Under these conditions, an increase in NaCl concentration up to 600 mM provoked a rise in fura2 signal (Fig. 3a). This indicated that the change in cytosolic free Ca^{2+} concentration originated from within the neutrophils. This also suggested that extracellular Ca^{2+} was not the sole source of the Ca^{2+} signal. However, when NaCl was raised to 1200 mM, there was evidence for cell breakage. The fura2 signal rose transiently before suddenly falling to a low level (Fig. 3a), suggesting that the initial elevation in cytosolic free Ca^{2+} was followed by cell lysis and release of fura2 into the low Ca^{2+} extracellular environment.

3.3.2. Cytosolic free Ca^{2+} rise is reversed by restoring isotonicity

In order to confirm that cell lysis did not constitute a significant part of the increase in fura2 signal, the effect of restoring isotonicity was investigated. Neutrophils, in which cytosolic free Ca^{2+} was elevated by increasing the extracellular osmolarity, were placed into isotonic medium by centrifugation and re-suspension. During the time required to do

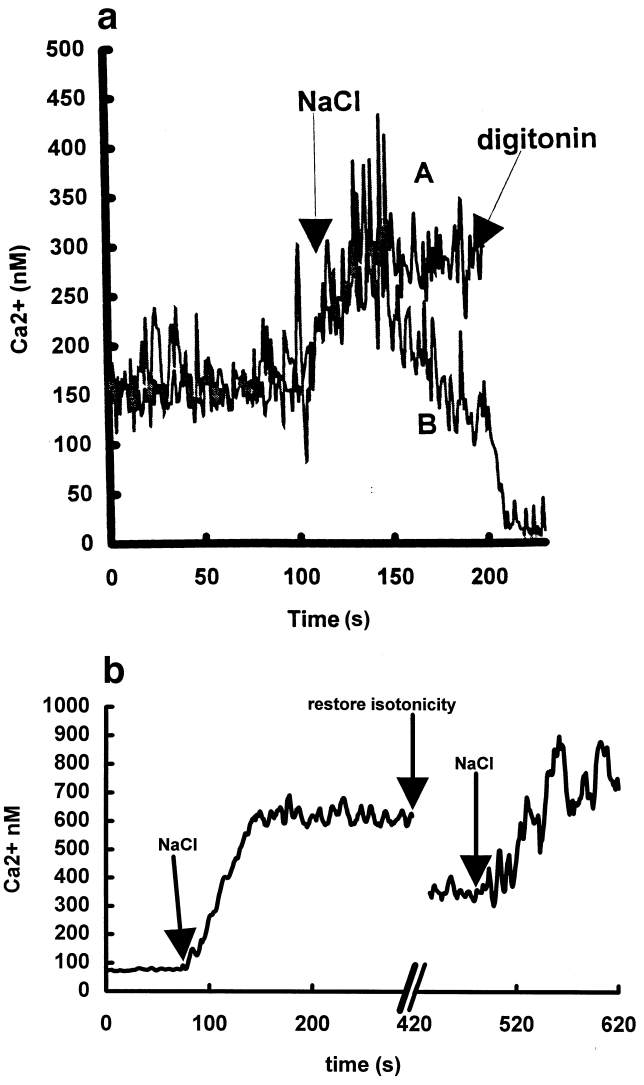

Fig. 3. Evidence that cytosolic free Ca^{2+} change is not due to cell lysis. (a) The change in cytosolic free Ca^{2+} concentration in human neutrophils in the absence of extracellular Ca^{2+} (EGTA, 1 mM) induced by the addition of NaCl (A, 600 mM and B, 1200 mM, added at the arrow) is shown. Digitonin (150 μM) was added at the end of the experiment to demonstrate the effect of total lysis. It is seen that in trace B, the addition of digitonin results in a rapid fall in measured cytosolic free Ca^{2+}. The effect of digitonin on trace A is not shown as this merely elevates the fura2 signal to R_{max} and is thus unquantifiable in terms of Ca^{2+}concentration. (b) The change in cytosolic free Ca^{2+} concentration in human neutrophils in the presence of extracellular Ca^{2+} is shown. NaCl (600 mM) was added at the first arrow, osmolarity was restored by centrifugation and re-suspension (at the second arrow) and NaCl (600 mM) added for a second time at the third arrow.

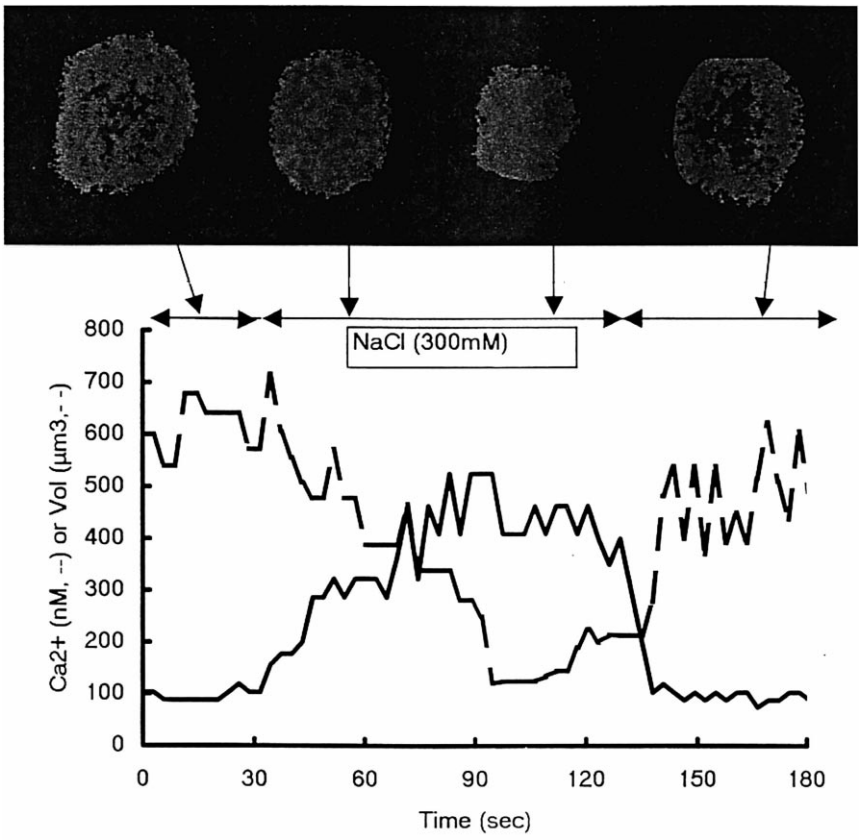

Fig. 4. Cytosolic free Ca^{2+} imaging. Loosely adherent fura2-loaded neutrophils were perfused with media of normal and elevated osmolarity while Ca^{2+} imaging was performed. The lower graph shows the cytosolic free Ca^{2+} concentration (solid line) and the neutrophil volume (broken line) calculated from serial images, during the change in osmolarity induced by addition of NaCl (300 mM) and restoration of normal osmolarity as indicated by the arrowheads. The images above the graph are representative of the data and are derived from the time points indicated by the arrows to the graph below. The images are pseudo-coloured for cytosolic free Ca^{2+} concentration, where dark to light represents low to high Ca^{2+}, and the image size is determined by the axis which is calibrated in micrometres.

this (i.e., approximately 3 min), the cytosolic free Ca^{2+} fell from approximately 800 to 300 nM (Fig. 3b). When these neutrophils were subjected to a second osmolarity increase (by 600 mM NaCl), there was a second increase in cytosolic free Ca^{2+}, to 85% of the initial response (Fig. 3b). The reversibility and persistence of responsiveness confirmed that the increase in osmolarity ($\leq$600 mM NaCl) did not result in significant cell lysis.

3.3.3. *Visualisation of cytosolic free Ca^{2+} change during osmotic challenge*

Imaging cytosolic free Ca^{2+} in individual neutrophils during increases in extracellular NaCl (up to 600 mM) provided evidence for an increase in cytosolic free Ca^{2+}, rather than for cell lysis. This direct visualisation showed that the fura2 signal originated from within the neutrophils (Fig. 4). However, when exposed to NaCl (1200 mM), elevated free Ca^{2+} was observed within the cell, but was followed within 1–8 s by loss of the image. This loss of image was the result of lysis and release of fura2 from the cell, such that the intensity fell below the threshold set for the data acquisition. The release of fura2 at high osmolarity was consistent with the data observed in experiments performed in the absence of extracellular Ca^{2+} (Fig. 3b).

3.4. *Relationship between cell volume and cytosolic free Ca^{2+} change*

Measurement of neutrophils by bright field micros-

copy showed that the diameter of the cell decreased by approximately 1–2 μm during the experimental change in extracellular osmolarity. In order to establish the temporal relationship between the decrease in neutrophil diameter and the increase in cytosolic free Ca^{2+} concentration, the threshold value for the acquisition of ratio-fura2 data was set so that the 'Ca^{2+} image' of the cell was the same size as the image of the cell as it appeared by bright-field microscopy. Under these imaging conditions, it was demonstrated that both the diameter and the cytosolic free Ca^{2+} concentration in loosely adherent spherical neutrophils changed synchronously (Fig. 4). Since the cell volume decreased, it was concluded that the rise in free Ca^{2+} concentration may have resulted from neutrophil water loss.

3.5. Mechanism of cytosolic free Ca^{2+} rise

In order to investigate whether the Ca^{2+} rise involved IP_3 production, the phospholipase C inhibitor, U73122 [16], was used. When neutrophils were pre-treated with the inhibitor, physiological Ca^{2+} signalling by f-Met-Leu-Phe was inhibited but the response to osmotic change (NaCl, 600 mM) was unaffected (Fig. 5a). As neither the physiological route nor the response to osmotic change was inhibited by an inactive analogue of the phospholipase C inhibitor, U73343, it was concluded that IP_3 production was not involved in the osmotically induced Ca^{2+} signal. In order to investigate whether the reduction in cell volume was the result of cytoskeletal contraction, neutrophils were pre-treated with the microfilament inhibitor, cytochalasin B. Under these conditions, an osmotically-induced Ca^{2+} rise was still provoked (Fig. 5b). It was therefore concluded that an intact cytoskeleton was not required for the osmotic Ca^{2+} signal.

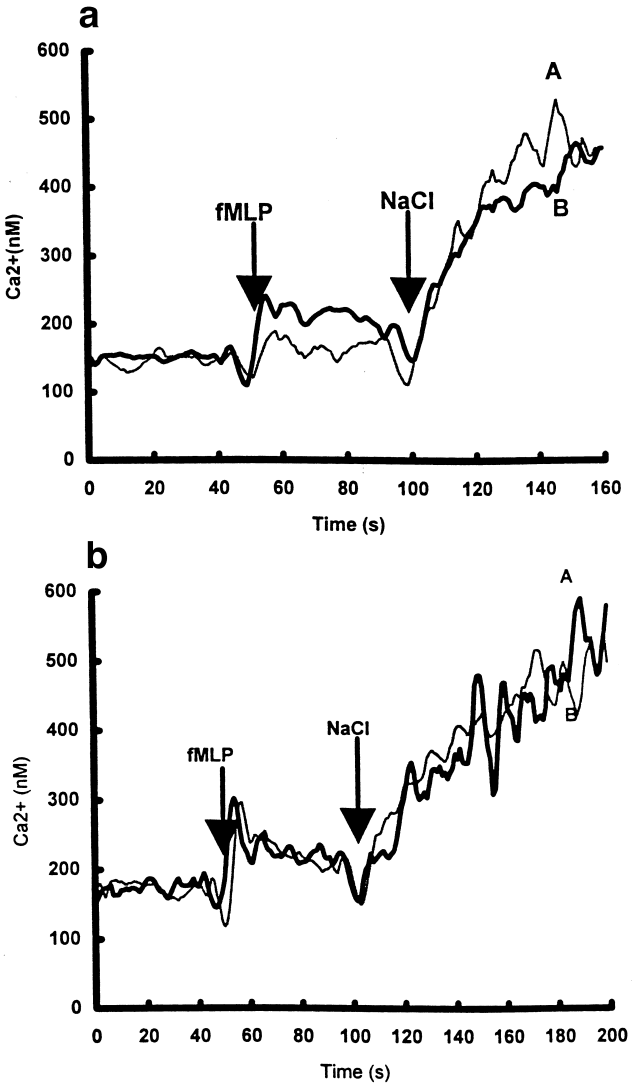

Fig. 5. Effect of inhibitors on osmotically induced cytosolic free Ca^{2+} changes. (a) The change in cytosolic free Ca^{2+} concentration in response to f-Met-Leu-Phe (1 μM) and NaCl (600 mM) added at the arrows, in neutrophils pre-incubated with U73122 (A) or its inactive analogue, U73443 (B). (b) The change in cytosolic free Ca^{2+} concentration in response to f-Met-Leu-Phe (1 μM) and NaCl (600 mM) added at the arrows, in control neutrophils (A) and those treated with cytochalasin B (5 μg/ml, B).

4. Discussion

In this paper we have demonstrated that increases in osmolarity cause a physiologically significant rise in cytosolic free Ca^{2+} concentration within human neutrophils. The data is consistent with a mechanism where by osmotic water loss from the cytosol leads to a reduction in neutrophil volume with a consequent elevation in the concentration of cytosolic constituents, including Ca^{2+} ions. This finding may provide an explanation for some previously reported effects of hyperosmolarity over the same range on neutrophil behaviour. For example, the ability of neutrophils to kill *Pseudomonas aeruginosa or Staphylococcus aureus* was impaired by osmolarities greater than 500 mosmol [2,17], and superoxide produc-

tion and myeloperoxidase secretion from neutrophils in response to a Ca^{2+}-dependent stimulus (f-Met-Leu-Phe) was reduced by changes in osmolarity whereas the response to a Ca^{2+}-independent stimulus (phorbol myristate acetate) was unaffected [18]. These and other reported effects are explicable as a consequence of disturbance of cytosolic free Ca^{2+} homeostasis and increased Ca^{2+} pumping. In neutrophils, which have few or no mitochondria, such pumping may have severe consequences for the cellular ATP level and thus on ATP-dependent activities. It is thus important to note that it has previously been reported that hyperosmotic conditions reduce cellular ATP in these cells [19].

There were several mechanisms by which osmotic changes could trigger an elevation of cytosolic free Ca^{2+}. The elevation of cytosolic free Ca^{2+} in the absence of extracellular Ca^{2+} (Fig. 3a) could have been signalled via an established route, namely via phospholipase C activation and IP_3. However, this possibility was eliminated by the use of the phospholipase inhibitor U73122 which failed to inhibit the osmotically induced Ca^{2+} signal despite inhibiting that provoked by f-Met-Leu-Phe (Fig. 5a). Simultaneous measurement of cell size with cytosolic free Ca^{2+} concentration revealed a close temporal relationship between the two events, suggesting a causal link. Previous work has shown that elevation of cytosolic free Ca^{2+} concentration by other stimuli does not cause neutrophil shrinkage. Indeed, in the absence of stimulation, elevation of cytosolic free Ca^{2+} by release of caged Ca^{2+} caused neutrophil spreading [20]. Rather than cell shrinkage being the *result* of a rise in cytosolic free Ca^{2+} concentration, it therefore seemed more likely to be the *cause* of the rise in cytosolic free Ca^{2+} concentration. As cell shrinking was not dependent on an intact microfilament system (Fig. 5b), it was possible that cell shrinkage was a result of osmotic water loss. If this were the case, the concentration of all cytosolic constituents would rise during cell shrinking. By reducing the diameter of a spherical neutrophil by approximately 4 μm, the consequent volume reduction would give rise to an increase in the concentration of all constituents within the cell by approximately 460% (i.e., $100\times R^3/(R-d)^3$, where R is the initial cell diameter and d is the reduction in diameter). However, the Ca^{2+} buffering and homeostatic mechanisms within the cell might be expected to reduce the change in cytosolic free Ca^{2+} concentration because the Ca^{2+} buffering capacity of the untreated neutrophil is estimated to be about 1000–3000:1 [21]. Surprisingly, from the data shown in Fig. 4, the theoretical increase in the concentration of cell unbuffered constituents was similar to the observed cytosolic free Ca^{2+} increase (being 5.8-fold and 5.9-fold, respectively). This suggests that the cytosolic free Ca^{2+} rise occurred in the absence of effective buffering. The Ca^{2+} buffering organelles within the cell may therefore also have been osmotically compromised and rendered inoperative. This would provide an explanation for the dramatic rises in cytosolic free Ca^{2+} concentration which were observed in the absence of extracellular Ca^{2+} and may have arisen from a redistribution of cellular Ca^{2+} between storage organelles and the cytosol. This proposal also provides an explanation for the results of Kazilek et al. [5]. Although cytosolic free Ca^{2+} concentration was not quantified by these authors, the basal indo-1 fluorescence was increased after hypertonic treatment and the f-Met-Leu-Phe-induced Ca^{2+} signal was reduced [5]. Both phenomena could result from ineffectual Ca^{2+} storage under hypotonic conditions, providing a source of Ca^{2+} for the elevation in resting cytosolic free Ca^{2+} concentration and a reduced release pool upon stimulation.

We thus propose that any conditions in which the neutrophil volume were reduced by water loss would result in the same intracellular events which lead to an elevation of cytosolic free Ca^{2+} concentration. In the kidney and some inflammatory sites, the effect of osmolarity may raise cytosolic free Ca^{2+} to a concentration sufficient to trigger cell activity [1–4]. In the lung airway fluid, water loss from neutrophils may also occur as a result of desiccation stress resulting from evaporation in the lung [22]. It is, however, unlikely that the abnormal osmolarity of the airway surface fluid which is reported in CF [23,24] would provoke the effects on cytosolic free Ca^{2+} concentration reported here. Elevation of cytosolic free Ca^{2+} concentration within neutrophils in hyperosmotic sites within the body may trigger the release of proteases, activate the neutrophil oxidase and lead to tissue damage. Furthermore, elevated cytosolic free Ca^{2+} within neutrophils inhibits their ability to phagocytose and migrate [25]. A large population

of ineffective, yet activated neutrophils at some sites may thus have a role in the aetiology of some inflammatory diseases. It is now necessary, therefore, to examine whether extracellular hyperosmolarity plays a part in the neutrophil-mediated degradative process in disease.

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